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SHORT COMMUNICATION

Limitations in the Use of the 340 nm Absorbance Maximum of Reduced Nicotinamide Adenine Dinucleotide Phosphate for the Determination of Oxidation Rates and Stoichiometry during Rat Hepatic Microsomal Metabolism

MILDRED K. BUENING AND MICHAEL R. FRANKLIN

Department of Pharmacology, University of Utah, Salt Lake City, Utah 84132

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SUMMARY

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During rat hepatic microsomal oxidative metabolism NADPH not only is oxidized to $NADP^+$ but is also cleaved to reduced nicotinamide mononucleotide. The 340 nm absorbance maximum of NMNH interferes with the use of the 340 nm absorbance as a measure of NADPH oxidation. The pyrophosphatase responsible for NADPH cleavage can destroy 30% of the added NADPH in the absence of exogenous mixed-function oxidase substrates in microsomes from phenobarbital-treated rats. It is inhibited about 75% by 2 mM 5′-AMP and 25 mM sodium pyrophosphate and about 30% by 10 mM sodium fluoride. Using pyrophosphatase inhibitors, and allowing for the turbidity enhancement of the 340 nm absorbance, the stoichiometry of NADPH oxidized to oxygen consumed approaches 1:1, especially in the presence of exogenous mixed-function oxidase substrates.

Studies of stoichiometry during microsomal mixed-function oxidations would be greatly facilitated if changes in absorbance at 340 nm in microsomal suspensions could be directly related to NADPH oxidation rates. With the Aminco DW-2 UV-VIS spectrophotometer, absorption spectra of turbid solutions in the ultraviolet region can be resolved, and direct measurement of the 340 nm absorbance of reduced pyridine nucleotides in microsomal suspensions is possible.

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However, limitations in the use of the 340 nm absorbance maximum for the determination of oxidative rates of NADPH in stoichiometric studies must be considered.

During NADPH oxidation in rat hepatic microsomal suspensions a portion of a reduced pyridine nucleotide was refractory to oxidation (Fig. 1A). A significant residual absorbance at 340 nm remained after all changes in the absorbance maximum had ceased. The amount of 340 nm absorbance which remained was proportional to the

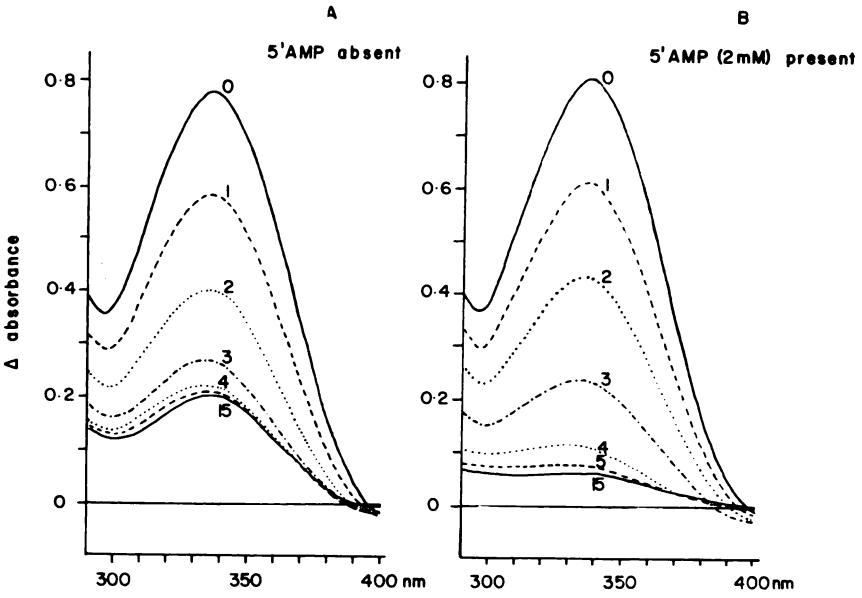

FIG. 1. *Disappearance of NADPH absorbance at 340 nm in microsomal suspensions*

Liver microsomes from rats treated with phenobarbital were suspended at 2 mg of protein per milliliter (cytochrome P-450, 4.8 μM) in 50 mM Tris-chloride buffer (pH 7.4) containing 150 mM KCl and 10 mM $MgCl_2$. The absorbance changes were monitored with an Aminco DW-2 UV-VIS spectrophotometer in the split-beam mode. NADPH was added to the contents of the sample cuvette, and the recording was initiated (curve at 0 min). Additional recordings were made at 1, 2, 3, 4, 5, and 15 min. A. The absorbance changes in the absence of 5'-AMP. B. Changes in the presence of 2 mM 5'-AMP.

concentration of NADPH added to the microsomal suspension initially.

Rat liver microsomes contain a pyrophosphatase capable of cleaving NADPH to reduced nicotinamide mononucleotide and 2',5'-AMP (1, 2). This enzyme is inhibited by 5'-AMP, sodium pyrophosphate, and sodium fluoride (2). When the oxidation of NADPH was examined in the presence of these pyrophosphatase inhibitors, residual 340 nm absorbance was decreased by 75% in the presence of 2 mM 5'-AMP or 25 mM sodium pyrophosphate, and by 30% in the presence of 10 mM sodium fluoride (Fig. 2). Since NMNH has an absorbance maximum at 340 nm, it cannot be distinguished spectrophotometrically from NADPH. Thus, when pyrophosphatase is active in microsomal preparations, significant amounts of NADPH may be cleaved to NMNH and no change in absorbance at 340 nm will be observed (3). The discrepancy between determination of NADPH by enzymatic methods and by spectrophotometric methods can be accounted for by the formation of NMNH from NADPH (4).

Although a range of concentrations of the pyrophosphatase inhibitors was examined, the residual 340 nm absorbance could not be completely eliminated. After microsomal NADPH oxidation in the presence of pyrophosphatase inhibitors, the absorbance at 340 nm was found to be ill-defined and broad, with no specific maximum at 340 nm (Fig. 1B). In an attempt to reduce this residual 340 nm absorbance, mixed-function oxidase substrates were included in the incubation (Fig. 3). In the absence of pyrophosphatase inhibitors the presence of the substrates reduced the residual 340 nm absorbance, as expected, since the more rapid oxidation of NADPH drains a larger percentage of the NADPH through this pathway, leaving less available for the pyrophosphatase. In the presence of a pyrophosphatase inhibitor, however, the substrates had no effect on the residual 340 nm absorbance, even though different NADPH oxidation rates were observed with the various

substrates. The identity of the very small 340 nm absorbance after NADPH oxidation in the presence of inhibited pyrophosphatase activity remains obscure. Our inability to alter its concentration either by increasing inhibitor concentration or by increasing NADPH oxidation rates suggests that in fact 100% inhibition of pyrophosphatase may be obtained with 2 mM 5′-AMP or 25 mM sodium pyrophosphate.

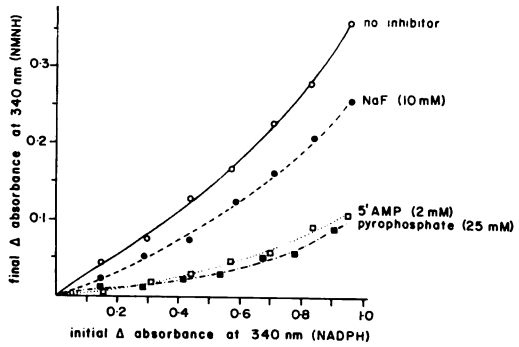

FIG. 2. *Effect of pyrophosphate inhibitors on residual 340 nm absorbance following microsomal NADPH oxidation*

The initial 340 nm absorbance upon NADPH addition and the residual absorbance 10 min later were determined as described in Fig. 3 for a range of NADPH concentrations in the presence of several pyrophosphatase inhibitors.

The rather surprising magnitude of pyrophosphatase activity in microsomes from phenobarbital-treated rats raised the possibility that induction by phenobarbital may cause an increase in pyrophosphatase activity. However, microsomes from untreated rats had even higher levels of pyrophosphatase activity (Fig. 4). Jeffery and Mannering (4) also observed that microsomal pyrophosphatase activity was not induced by treatment with phenobarbital. The pyrophosphatase inhibitor 5′-AMP appeared to have a greater inhibitory action in microsomes from control animals compared with those which had been treated with phenobarbital. The explanation is probably not a difference in enzyme inhibition, but rather that microsomes from phenobarbital-treated rats contain more cytochrome P-450 and therefore are more capable of oxidizing NADPH during the metabolism of endogenous substrates. Thus, in microsomes from phenobarbital-treated rats, more of the

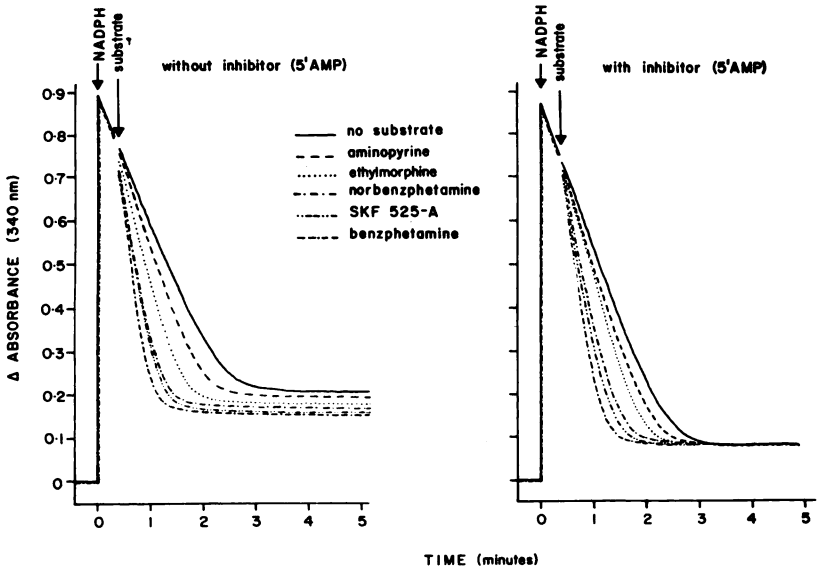

FIG. 3. *Effect of mixed-function oxidase substrates on microsomal oxidation of NADPH*

Phenobarbital-treated rat microsomes (cytochrome P-450, 2.44 nmoles/mg of protein) were suspended in buffer as described in Fig. 1. Absorbance changes at 340 nm were monitored after the addition of NADPH (126 μM) and mixed-function oxidase substrate (100 μM) to samples in both the absence and presence of the pyrophosphatase inhibitor 5′-AMP (2 mM).

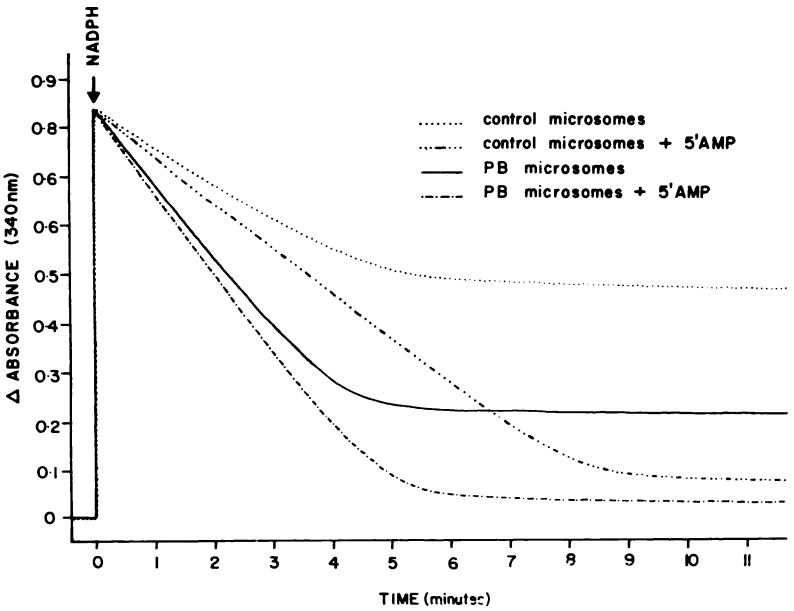

FIG. 4. *Comparison of disappearance of 340 nm absorbance of NADPH in microsomes from untreated and phenobarbital-treated rats*

Microsomes from untreated rats and phenobarbital (PB)-treated rats (cytochrome P-450, 1.24 and 2.40 nmoles/mg of protein, respectively) were suspended in buffer as described in Fig. 1. Absorbance changes at 340 nm were monitored after the addition of 110 μM NADPH to the sample cuvette. The experiments were performed in both the presence and absence of 2 mM 5'-AMP.

NADPH is directed through oxidative reactions rather than the pyrophosphatase reaction.

With the NADPH utilization routes more clearly defined, the stoichiometry of NADPH in relation to oxygen could be investigated. However, in microsomal suspensions direct measurement of NADPH concentration by the absorbance at 340 nm was hindered by "turbidity enhancement" (5). The greater the microsomal protein concentration (i.e., the more turbid the suspensions), the greater was the absorbance obtained from addition of a given amount of NADPH. A microsomal concentration of 2 mg of protein per milliliter produced up to 17% enhancement in the range of NADPH concentrations used. Allowance was made for this turbidity enhancement in the stoichiometric studies by calibration of NADPH in clear (buffer) solution.

The stoichiometry of NADPH oxidized to oxygen consumed was investigated in the presence and absence of ethylmorphine both with and without 5'-AMP (Fig. 5). The amount of oxygen consumed in relation to NADPH was less in the absence than in the presence of pyrophosphatase inhibitors (lower graphs). This observation can be correlated with the larger amounts of NADPH observed as the 340 nm residual absorbance of NMNH (upper graphs). Thus the presence of pyrophosphatase inhibitors raised the ratio of NADPH oxidized to oxygen consumed from 0.83 to 0.93 in the absence of ethylmorphine (left side) and from 0.95 to 0.99 in the presence of ethylmorphine (right side). The proximity of this ratio to unity confirms the hypothesis that the pyridine nucleotidase in rat liver microsomes has little effect on NADPH and mainly affects the oxidized form of the nucleotide. Nicotinamide blocks the action of the nucleotidase (6) but does not block pyrophosphatase (1, 2).

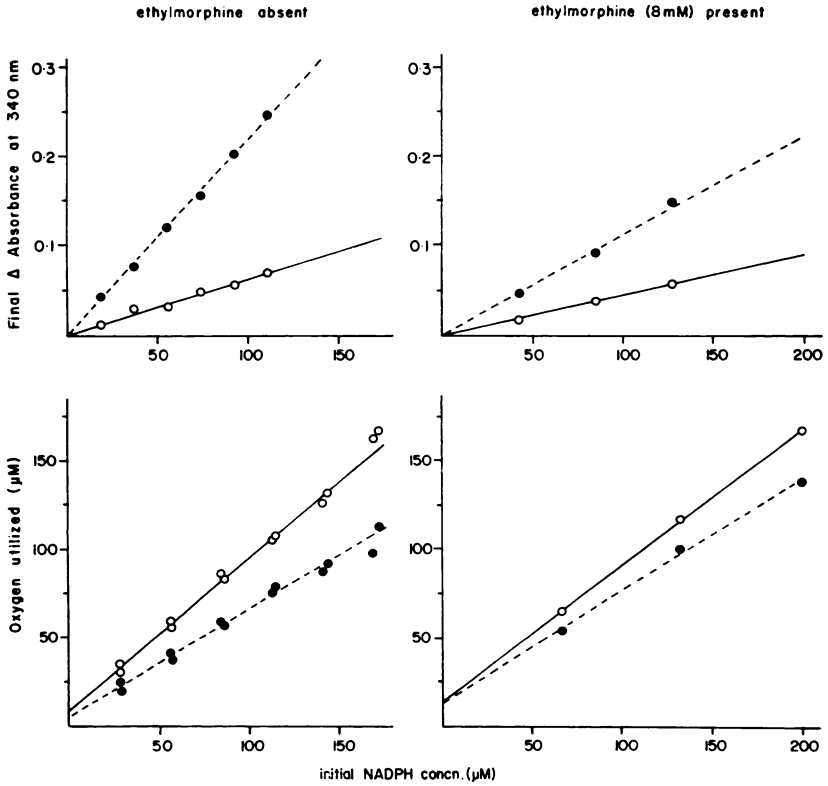

FIG. 5. *Stoichiometry of microsomal NADPH oxidation and oxygen consumption in the presence and absence of ethylmorphine and a pyrophosphatase inhibitor*

Phenobarbital-treated rat microsomes (cytochrome P-450, 2.36 nmoles/mg of protein) were suspended in buffer as described in Fig. 1. Various amounts of NADPH were added to samples in the presence and absence of 8 mM ethylmorphine both with (O——O) and without (●- - -●) 2 mM 5′-AMP. Absorbance changes at 340 nm were monitored for 10 min to give both the initial and final absorbance. A membrane-coated Clark oxygen electrode was used to determine oxygen consumption in a similar set of samples.

In stoichiometric studies performed in rat liver microsomal suspensions, extreme caution is necessary in interpreting the results when the absorbance at 340 nm is used as the criterion of NADPH concentration. The use of a pyrophosphatase inhibitor in these studies would seem to be recommended.

The formation of NMNH from NADPH also raises an additional complication in studies of reduced pyridine nucleotide requirements for cytochrome P-450-catalyzed, mixed-function oxidations (7, 8), and the full effect of NMNH formation from, and action on, the oxidation of NADH and NADPH needs to be investigated.

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